#926

CARDIOVASCULAR

Comm.
Dr. Bing
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant — Date. July 19, 1973
(Use extra pages as needed)

RECEIVED
JUL 26 1973

1. Principal Investigator (give title and degrees)

Budh Dev Bhagat, Ph.D.
Professor of Physiology

2. Institution & address:

St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, Missouri 63104

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

Effect of Smoking on the Cardiovascular System in Experimental Hypertension

5 Proposed starting date: October 1, 1973

6 Estimated time to complete: Three years

7. Brief description of specific research aims:

See proposal

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8. Brief statement of working hypothesis:

See proposal

9. Details of experimental design and procedures (append extra pages as necessary)

See proposal

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space: Our laboratory and office space (approximately 600 squ.ft.) is well equipped with all the standard facilities. In addition, a cold room radioisotope room, animal room and machine shop are also available.

Equipment: In addition to standard laboratory equipment, such as glassware and other apparatus, the following items are available for our use: Grass stimulator, spectrofluorometer (Aminco-Bowman), mechanical shaker, and International portable refrigerated centrifuge.

In addition, facilities of the Physiology Laboratory of the Department of Gynecology and Obstetrics (Dr. Cavanagh) will be available for use. The facilities are adequate for the sterile operative procedures required.

Animal Research Space: Ample animal and laboratory space is available in the new renovated Animal Care Facility at St. Louis University Medical Center to permit proper conduct of these studies. These quarters are under the direction of a veterinarian who quarantines and conditions animals prior to use in experiments.

Library: An excellent medical library supports the research service. It includes over 5,000 volumes and regularly subscribes to 189 scientific periodicals. An excellent interlibrary loan system with the four Universities and two medical societies in our area gives us ready reference material promptly. The Yalem Computer Center of St. Louis University is readily available for the processing of data and gives a priority to medical research.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append):

See page attached

13. Publications: (five most recent and pertinent of investigator(s), append list, and provide reprints if available).

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CURRICULUM VITAE

BUDH DEV BHAGAT, Ph.D.

Born: India, January 1, 1926; U.S. Citizen

Education:

Ph.D., Pharmacology, (Faculty Medicine),
London University, 1961

Postdoctoral in Pharmacology,
University of Wisconsin Medical School, 1962

Postdoctoral in Pharmacology,
University of Minnesota Medical School, 1963

Faculty Appointments:

Assistant Professor, Department of Pharmacology,
Howard University Medical School, 1964-66

Assistant Professor, Department of Pharmacology,
New York Medical College, 1966-68

Associate Professor, Department of Physiology
Associate Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1968-71

Professor, Department of Physiology
Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1971-

Major Research Interests:

Autonomic Nervous System, Neurotransmitter, Cardiovascular

Committee Appointment:

Member - Advisory Board for "Neurosciences Research," Academic Press

Publications:

Approximately 169 publications to date.

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FIVE PERTINENT PUBLICATIONS

1. B. Bhagat. Effects of dermic administration of nicotine and storage and synthesis of noradrenaline in rat brain. Br. J. Pharmac. 38: 86-92, 1970.

2. B. Bhagat. Influence of chronic administration of nicotine on the turnover and metabolism of noradrenaline in the rat brain. Psychopharmacologia 18: 325-332, 1970.

3. B. Bhagat, T. Bayer and C. Lind. Effect of chronic administration of nicotine on drug-induced hypnosis in mice. Psychopharmacologia 21: 287-293, 1971.

4. B. Bhagat and M.W. Rana. Effect of chronic administration of nicotine on the concentrations of adrenal enzymes involved in the synthesis and metabolism of adrenaline. Br. J. Pharmac. 43: 250-251, 1971.

5. Ping-lung Chang, B. Bhagat and John J. Taylor. Effect of chronic administration of nicotine on acetylcholinesterase activity in the hypothalamus and medulla oblongata of the rat brain. An ultrastructural study. Brain Res. 54: 75-84, 1973.

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R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested).	% time	Amount
B. Bhagat, Ph.D.	20%	none
1 Post-doctoral fellow (to be employed)	100%	REDACTED
Technical		
1 Technician (to be employed)	100%	REDACTED
Animal Caretaker	50%	
Fringe Benefits		
Sub-Total for A		REDACTED
B. Consumable supplies (by major categories)		
Radioactive material		1,000.
Drugs and chemicals		1,000.
Animals		2,500.
Maintenance of equipment		400.
Sub-Total for B		4,900.
C. Other expenses (itemize)		
Travel to attend National meetings		400.
Photographic material		200.
Reprint costs		200.
Sub-Total for C		800.
Running Total of A + B + C		REDACTED
D. Permanent equipment (itemize)		
NONE		
Sub-Total for D		
E. Indirect costs (15% of A+B-C) (exclusive of fringe benefits)		3,930.
Total request		REDACTED

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	4,900.	800.	---	4,072.	REDACTED
Year 3		4,900.	800.	---	4,233.	

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16. Other sources of financial support.
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Budh D. Bhagat

Signature B. Bhagat Date

Telephone (314) 664-9800 412
Area Code Number Extension

Checks payable to

St. Louis Univ. School of Medicine

Mailing address for checks

Controller, 221 N. Grand

St. Louis, Missouri 63103

Responsible officer of institution

Typed Name George E. Thoma

Title Asst. Vice Pres. & Research Administrator

Signature G. E. Thoma Date 7/23/73

Telephone (314) 865-2288 541
Area Code Number Extension

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PROPOSAL

Budh D. Bhagat

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SUMMARY

Cigarette smoking is suggested to be one of the major hazards in the United States. It is implicated in cardiovascular diseases. The present proposal is to conduct a study to throw light on the mechanism of action of smoking on the cardiovascular system in experimental hypertension. For this purpose we will measure endogenous catecholamines, accumulation of ^{3}H-norepinephrine, the activity of enzymes involved in synthesis and degradation, and the turnover rate of norepinephrine in the heart, adrenal galnds, and blood vessels, and determine the reactivity, in vitro, of the vascular smooth muscle. These studies will be conducted not only during smoking, but also during periods of withdrawal from cigarette smoking. An attempt will be made to correlate the biochemical changes in the cardiovascular system with the onset and degree of initial and subsequent hypertension. Finally, the effects of various drugs on hypertension will be determined.

It is our belief that this combined physiological and biochemical study may elucidate the role of smoking in the acceleration of cardiovascular diseases.

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INTRODUCTION AND SPECIFIC AIMS

There is enough evidence to suggest that cigarette smoking can contribute to the development of cardiovascular disease and particularly to death from coronary heart disease. Life expectancy among young men is reduced by an average of 7 to 8 years in heavy (over two packs a day) cigarette smokers and an average of 4 years in light (less than one-half pack a day) cigarette smokers. No substantial evidence has appeared to refute these forecasts.

While the exact mechanisms involved in the pathological effects of smoking are not known, the evidence suggests that cigarette smoking constitutes one of the major health hazards in the United States as well as in other parts of the world.

In normotensive persons, the intensity of sympathetic stimulation of the heart and blood vessels varies greatly with posture, activity, emotional state, physical conditioning and cardiovascular health. It influences venous capacitance, heart rate, myocardial contractility, as well as cardiac output and arteriolar resistance, the determinants of mean arterial pressure. Arterial pressure appears to be no less labile in hypertensive patients. This suggests that their sympathetic activity is also highly variable. This is further supported by the fact that the blood pressure of hypertensive men and animals is often lowered by the administration of drugs which alter the physiological disposition of NE, pointing toward substantial participation of this amine in the maintenance of high blood pressure. In addition, the increased vascular reactivity observed in some forms of human hypertension (Goldenberg et al., Am. J. Med. 5: 792, 1948) and experimental hypertension (Raab, Am. J. Cardiol. 4: 752, 1959) suggests that in these conditions there is either an impaired inactivation of amines or increased sensitivity of the effector cells.

Norepinephrine (NE) in the tissue innervated with sympathetic nerve endings is inactivated by at least three mechanisms: a) uptake and storage in nerve terminals, b) o-methylation by catechol-o-methyl transferase (COMT) and c) oxidative deamination by monoamine amine oxodase (MAO). Inactivation by uptake of NE is more important than inactivation by metabolism. In support of this is the observation that physiological effects of injected NE are rapidly terminated, even after both MAO and COMT are inhibited. Any drug or condition that prevents uptake or binding of NE would allow an increased amount of free catecholamine to remain in the vicinity of receptors, resulting in apparent supersensitivity to NE. Such a reduction in the myocardial accumulation of H^3-NE in experimental hypertension was actually demonstrated by DeChamplain et al (Life Science, 5: 2283, 1966).

Biochemical evidence of altered sympathetic nerve function has been reported in essential hypertension (Brunjes, 5: New Eng. J. Med. 271: 120, 1964).

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Abnormalities of tissue catecholamine metabolism in rabbits made hypertensive by complete denervation of the carotid sinuses and aortic arch (DeQuattro et al, Cir. Res. 24: 545, 1969) have been reported.

Rats made hypertensive with deoxycorticosterone (DOCA) and sodium have shown a hyperactivity of the sympathetic fibers and adrenal medulla. These rats seem to release more norepinephrine from the storage granules. This finding is reflected in lower endogenous norepinephrine levels, in the smaller proportion of ^{3}H-norepinephrine in the particulate as compared with the supernatant and in the increased excretion of norepinephrine and the deaminated and o-methylated metabolites in the kidney and urine (De Champlain et al., Cir. Res. 23: 479, 1968).

The spontaneously hypertensive rat (SHR) is another model of human essential hypertension (Okamoto and Aoki, Jap. Cir. J. 27: 282, 1963). It has been found that in the heart of SHR, norepinephrine turn-over rate was reduced in porportion to the rise in systolic blood pressure (Nakumura et al., Naunyn-Schmiedebergs Arch. Pharmak 271: 157, 1971).

Since there are alterations in the catecholamine patterns in the hypertensive patients and animals; since smoking is implicated in the development of cardiovascular diseases and particularly to death from coronary heart diseases and since nicotine, the principal alkaloid of tobacco produces its actions by release of catecholamines from its storage sites, it is therefore considered necessary to determine the effect of smoking on the synthesis and disposition of norepinephrine in the cardiovascular tissues in experimental hypertension.

In our proposed study, rats, both normotensive and hypertensive, will be exposed to tobacco smoke under conditions comparable to those of human smoke exposure. We will examine the following tissues: adrenal gland, superior cervical ganglia, heart, aorta, superior mesenteric artery, renal arteries, abdominal (inferior) vena cava and mesenteric vein. Changes in catecholamine pattern will be determined at various intervals following treatment (smoking) and following periods of withdrawal from cigarette smoking.

Thus the aim of the present proposals are:

To determine whether chronic smoking alters the catecholamine pattern in normotensive animals.

To determine how chronic smoking affects the altered pattern of catecholamines in experimental hypertensive rats.

To develop a more detailed understanding of the altered rate of synthesis and utilization of neurohormones in the cardiovascular tissues. We will examine animals at specific times following the start of smoking and (once the maximum changes in the catecholamine pattern have developed) during the subsequent period of cessation of smoking. An understanding of these factors is essential to attempt to define the mechanism involved in the synthesis and metabolism of these neurohormones.

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[illegible]
(DeQuattro et al. Cir. Res. 24: 545, 1969) have been reported.

To determine whether onset and degree of initial and subsequent hypertension are augmented by smoking in experimental hypertension.

To determine whether or not the changes in the catecholamine pattern are related to any changes in function of the components in the cardiovascular system.

[illegible]

It has been found that in the heart of SH[illegible] norepinephrine turn over [illegible]

[illegible]

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EXPERIMENTAL PROCEDURE

1. Preparation of Animals: Rats weighing about 60 to 70 gm will be used throughout this study. Animals will be placed in cages which will be kept under similar conditions of lighting and humidity in a room maintained at a temperature within the range of 21.0 ± 0.5°C. Food and water will be supplied ad libitum. No more than 6 rats (unless otherwise required) will be housed in each cage, since it was observed that crowding of animals increased the tyrosine hydroxylase by 32%. All animals will be acclimatized to the new environment for a period of one week before they are subjected to any treatment.

2. Body Weight: Body weight will be recorded weekly.

3. Food and Water: Food and water intake will be measured daily and expressed per 100 gm of body weight.

4. Measurement of Systolic Blood Pressure: The systolic blood pressure will be measured weekly in unanesthetized animals using a pulse transducer applied to the tail. The blood pressure of rats anesthetized with pentobarbital (60 mg/kg) will be measured by cannulation of the left carotid artery through a statham strain gauge (Pd 23) transducer.

5. Sex Difference: Whether there is a sex difference in the effect of smoking experiments in the females will be compared with males. Some experiments will be performed on pregnant rats.

6. Representative Model for Essential Human Hypertension: Although a representative model for essential human hypertension is not yet available, the analysis of the factors determining the development of various forms of experimental hypertension may yield new insight in the pathogenesis of essential human hypertension and additionally lead to new therapeutic approaches.

Two models will be used. 1) DOCA-salt hypertensive rats, 2) genetically hypertensive rats (SHR).

1) Production of hypertension in rats: Rats weighing 80 to 90 g will be anesthetized with nembutal. Under aseptic conditions, the right kidney and adrenal gland will be removed. The rats will be made hypertensive by subcutaneous injections of a suspension of deoxycorticosterone pyralate 10 mg per week and 1% NaCl solution to drink ad libitum for periods of 5-7 weeks. Both control and hypertensive animals will be fed a regular laboratory diet.

2) Spontaneous hypertensive rats: In 1963, Okamoto and Aoki (Jap. Cir. J. 271: 157, 1963) produced, by selective inbreeding a strain of Wistar rats with a 100% incidence of "spontaneous" hypertension. They (both male and female) will be used when they have reached an age of 3-14 weeks. They will be matched for age and body weight with a normotensive Wistar and a control obtained by backcrossing the spontaneously hypertensive rats with normotensive Wistar rats.

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7. Effect of Chemical Sympathectomy on Development of Hypertension: Most of the investigators believe that excessive activity of the sympathetic nervous system contributes to the development and persistence of abnormal arterial pressure in patients with primary hypertension. It is therefore planned to study the effect of smoking on development of hypertension in rats pretreated with 6-OH dopamine which causes a long-lasting depletion of norepinephrine from sympathetically innervated organs as a result of an acute and selective degeneration of the sympathetic adrenergic nerve (Tanzer and Thoenen, Experientia (Basel) 24: 155, 1968).

Four litters of rats will be studied beginning at birth, two litters will receive weekly subcutaneous injections of 6-OH dopamine (100 mg/kg). After reaching a body weight of 100 g, all four litters will be weaned and undergo unilateral nephrectomy. One litter of rats treated with 6-OH dopamine and one of untreated animals will be given deoxycorticosterone (DOCA) and 1% NaCl for 5 weeks. The other litter of rats treated with 6-OH dopamine and one of the untreated rats will serve as controls. All animals will be fed a regular laboratory diet.

8. Exposure to Smoke: Animals will be conditioned for at least one week prior to smoke exposure. Rats will be inserted into the animal cone holder and placed on the operating machine without cigarette, three times each day for 10 minutes. Suitably conditioned animals will enter the cone holders voluntarily.

Animals losing weight generally more than one gram per day during the conditioning period will be discarded, since these animals will not survive a chronic exposure. Following one week's exposure without smoke rats will be adopted with smoke to cigarette-concentration smoke for 8 minute exposure, 3 times a day.

The Walton Horizon Smoke Exposure Machine (developed under contract by the Council for Tobacco Research, U.S.A.) will be used. It has a capacity to expose 12 young rats to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure.

Essentially smoke will be produced by "positive" puffing (blowing) meter air through a horizontally-held cigarette enclosed in a plastic dome during a timed two-second puff. The two-second puff interval is defined as the interval when the dome is in contact with the cigarette-holder plate. The average puff volume is defined as the average puff volume of smoke produced during the first eight puffs. The 35 ml is the average puff volume of smoke produced during the first eight puffs.

In the normal one-minute cycle of operation the two-second puff will be followed by a 15-sec. hold period, i.e., for a total exposure time of 17 sec. This will be followed by a 30 sec purge period to sweep out the smoke and a 13 sec rest period. The smoke will be pushed into a constant volume (384cc) smoke exposure chamber. Uniform mixing will be achieved with a mechanical mixer attached to one of the animal cone-holder plates.

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Animals (conditioned for at least one week prior to smoke exposure) will be held in cone-shaped holder and will breathe the exposure chamber contents with their noses just inside the smoke chamber. They will be removed from the cone holder promptly after exposure to avoid water loss due to sweating and the additional stress of excessive confinement.

Cigarettes: Kentucky reference cigarettes (IRI) with different levels of nicotine will be used. They will be equilibrated for at least 24 hr at 76 (± 2)°F to (± 2)% relative humidity atmosphere, by placing them unwrapped, with package opened into a dessicator (on wire mesh shelves) containing a 74% w/w glycerol-water solution in the bottom compartment. The cigarettes will be placed loosely into the chamber.

9. Pharmacological Studies: At various intervals of treatment the sensitivity of the cardiovascular system to selected drugs will be surveyed. These include norepinephrine, epinephrine, tyramine, tryptamine, isoproterenol, acetylcholine, atropine, mephentermine, methoxamine HCl, guanethidine, prostaglandin, propranolol. Chronotropic sensitivity as well as blood pressure responses will be determined.

Since the cardiac responses in the intact animal may be modified by reflex response an isolated atrial preparation will be used, thus eliminating their indirect action. Responses to sympathetic stimuli will be compared with those taken from untreated animals.

Reactivity of the vascular smooth muscle: Altered peripheral resistance of the vascular system is the characteristic of hypertension. We will, therefore, compare the reactivity in vitro of thoracic aortas from hypertensive animals with those from controls. The thoracic aorta will be used as an indicator of vascular reactivity because it can readily be prepared for the recording of pharmacologic responses, although the aorta exerts little, if any, effect on total peripheral resistance. Dose response curves to specific (norepinephrine, serotine and histamine) and non-specific (potassium chloride) smooth muscle contractile substances will be determined. This will provide information as to whether there is an alteration in reactivity of the smooth muscle after endotoxin, and if this alteration is due to changes in the responsiveness of the muscle itself or due to specific modification of the receptors. If all the agonsits are affected to about the same degree, then the mechanism responsible for this abnormality may be related to the intrinsic contractility of the muscle itself and not to a specific modification of receptors.

Catecholamines: In order to gain a better knowledge concerning uptake, storage, release and metabolism of catecholamines in the sympathetic nerve, neurotransmitter in the sympathetic nerve will be tagged with the radioactive catecholamine. Endogenous norepinephrine levels, rate of uptake of, binding and metabolism of H-norepinephrine in various tissues will be determined.

Turn-over rate of norepinephrine in tissue: Changes in turnover rate of tissue in catecholamines provides a more sensitive indication of sympathetic activity than do changes in the tissue concentrations of amine

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which might remain constant or even decline in spite of an increased rate of synthesis. Therefore, turn-over rate of norepinephrine in cardiovascular tissue will be determined according to steady-state kinetics (Brodie et al, J. Pharmacol. & Exp. Ther. 154: 493, 1966).

Tyrosine hydroxylase activity: The catecholamines, norepinephrine and epinephrine, are continuously being synthesized, released and metabolized. However, tissue catecholamines remain at a steady level characteristic of each organ. It appears that there is a dynamic balance between the rate of synthesis of norepinephrine and its disappearance.

The procedures which increase sympathetic nervous activity, such as exposure to cold or heat, exercise, -receptor blockade, thyroidectomy and electrical stimulation of sympathetic nerves, produce an increase in the synthesis of norepinephrine as a result of increased tyrosine hydroxylase activity. However, this effect occurs without an increase in the amount of enzyme. The increase in enzyme activity is due to release of tyrosine hydroxylase from the end-product inhibiton.

It is recently reported that in rats rendered hypertensive by carotid sinus denervation, the content of tyrosine hydroxylase in the heart was significantly greater than that observed in hearts of control animals (De Quattro, et al, Fed. Proc. 27: 240, 1968). Conceivably, chronically increased sympathetic nervous activity may lead to increased synthesis of tyrosine hydroxylase and an increased content of this enzyme in adrenergic nervous tissue. This phenomenon may be highly significant in the pathogenesis of disease states where an increased sympathetic nervous activity is a significant component.

The chronic increase in the sympathoadrenal activity induced by exposure to severe stresses has been shown to elevate adrenal tyrosine hydroxylase levles (Mueller et al, J.P.E.T. 169: 74-79, 1969).

Since smoking results in an increased sympathoadrenal activity, it will be interesting to measure tyrosine hydroxylase activity in various tissues.

Phenylethanol-N-Methyl Transferase (PNMT) in adrenal glands: PNMT is another enzyme which converts norepinephrine to epinephrine in the adrenal gland. The activity of PNMT increases in response to various conditions or treatments which increase the tyrosine hydroxylase activity. These include insulin-induced hypoglycemia (Patrick and Kirshner, Mol. Pharmacol., 7: 87, 1971), administration of reserpine (Bhagat et al, Br. J. Pharmac. 43: 819, 1971) or 6 hydroxydopamine (Thoenen et al, Biochem. Pharmacol. 19: 669, 1970) immobilization stress (Kvetnansky et al, Endocrinology 87: 744, 1970) and stress by prolonged isolation or by repeated exposure to cold. In all these conditions activation of the sympathoadrenal system and enhanced secretion of catecholamines are the common denominator. Since smoking and administration of nicotine results in an increased sympathoadrenal activity, it will be interesting to measure PNMT activity of the adrenal gland in the rat. We have already evidence that PNMT activity in adrenal gland is increased after chronic treatment with nicotine (Bhagat and Rana, Brit. J. Pharmac. 43: 250, 1971).

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The following parameters will be measured in the adrenal gland: 1) norepinephrine, 2) epinephrine, 3) TH activity, 4) MAO activity, 5) COMT activity, 6) phenylethanol-N-methyl transferase.

The following parameters will be measured in superior cervical ganglia: 1) norepinephrine, 2) TH activity, 3) MAO activity, 4) COMT activity.

The following parameters will be measured in heart and vascular tissues: 1) norepinephrine, 2) capacity to take up and accumulate ^{3}H-norepinephrine, 3) the rate of metabolism of ^{3}H-norepinephrine, 4) rate of conversion of ^{3}H--tyrosine to ^{3}H-norepinephrine, 5) MAO activity, 6) COMT activity, 7) TH activity.

All vascular tissues will be carefully cleaned of adhering tissue with forceps or a small nylon brush as described by Koletsky et al (Proc. Soc. Exp. Biol. Med. 102: 12-15, 1959). Microscopic examination of the vessels will be made to confirm that adhering tissues (connective tissue, fat and extravascular nerves) have been removed.

Histomorphological Changes: Tissues such as kidney, lungs, liver, heart, endocrine glands from experimental animals will be studied histologically using H & E and PAS stains; also ultrastructure of these tissues will be studied.

These studies will be carried out with collaboration of Dr. K. Christensen, Professor of Anatomy.

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METHODS

Isolated Atrial Preparation: The atria will be freed of ventricular muscle, connective tissue, fat and blood vessels; it will then be placed in a modified Tyrode's solution maintained at 34°C. A mixture of 95% oxygen and 5% carbon dioxide will be bubbled through the bathing fluid through a sintered glass plate at the bottom of the bath. The Tyrode's solution will have the following composition: NaCl, 0.9%; KCl, 0.04%; CaCl, 0.24%; $NaHCO_3$, 0.05%; glucose, 0.20%. The bicarbonate concentration employed will maintain the pH at approximately 7.4. The atria will be attached to a Grass force-displacement transducer; and isometric contractile force (resting tension of approximately 0.5 g) and rate of spontaneous contraction will be recorded by means of a Grass polygraph. The atria will be allowed to equilibrate at least 1 hour after being placed in the bath and will be washed repeatedly after each addition of the drug.

Left atrial strips driven electrically: The left atrium will be dissected from the heart and suspended in a (modified) Tyrode solution maintained at 34°C. It will be aerated with 95% and 5% CO_2. The lower end of the atrium will be tied to a plastic holder containing punctate electrodes. The upper end will be tied to a force-displacement transducer (Grass FT.03C) and contractions will be recorded on a Grass ink-writing oscillograph. Two atria (control and experimental) will be mounted in an organbath of 70 ml capacity. The atrium will be electrically driven via platinum electrodes, parallel to but not touching the tissue, with square-wave pulses of 5-msec duration, at frequency of 1/sec and above threshhold voltage. The resting tension on the atria will be 1.0 g. The atria will be allowed to equilibrate for 1 hour after being placed in the bath and will be washed repeatedly after each addition of the drug.

Dose-response curve to sympathomimetic amines and other drugs. Cumulative dose-response curves to sympathomimetic amines, 5-hydroxy-tryptamine, and histamine will be determined by a stepwise increase of the total concentration. The concentration will be increased as soon as the response to the preceding dose reaches the maximal point (i.e., at intervals of 1 to 4 minutes).

To measure the sensitivity of atria to amines, the log concentration of the amine will be plotted against per cent of the maximum response. From each individual dose-response curve, a concentration which caused 50% of the maximum response will be calculated. The ratio ED-50 of the preparation, made from baboons pretreated with endotoxin, over ED-50 of control is a measure of sensitivity.

Aortic strips: Spirally cut thoracic aortic strips will be prepared by the method of Furchgolt and Bhadrakom (J. Pharm. Exp. Ther. 108: 129, 1953). Each strip will be suspended in an isolated-organ bath (10 ml) containing a modified Krebs bicarbonate solution of the following composition (in gram per liter): KCl, 0.35; $CaCl_2$, H_2O, 0.37; KH_{2po4},

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0.16; $MgSO_4$, $7H_2O$, 0.29; NaCl, 6.9; $NaHCO_3$, 2.08 and glucose, 1.8. The temperature of the bath will be maintained at 37.5°C and the Krebs bicarbonate solution will be oxygenated with a mixture of 95 percent oxygen and 5 percent carbon dioxide. The strips will be subjected to an initial tension of 1 gram and will be kept in the organ bath for approximately 1 hour before drugs will be tested. Responses of the drugs will be measured isometrically with a force-displacement transducer and will be recorded on a polygraph as changes in tension in grams.

Chemical Methods

Animals will be killed by a blow on the head and decapitated. Various tissues will be rapidly removed, cleaned, frozen on dry ice and stored at 20°C prior to analysis.

1. Endogenous norepinephrine will be assayed by the method of Anton and Sayre (J.P.E.T. 133: 360, 1962). The method involves the selective absorption of catecholamines onto a constant amount of aluminum oxide, elution with a constant volume of perchloric acid and their measurement by the formation of fluorescent trihydroxyindole in the presence of potassium ferricyanide and alkaline ascorbare. To differentiate between epinephrine and norepinephrine, fluorescence is measured at 2 different pH's (pH 2-3 and pH 5-7). In the lower pH range, norepinephrine compared to epinephrine has a negligible fluorescence. Of the naturally occurring analogues of norepinephrine, only dopamine interferes, but this interference is reported to be relatively small. Samples will be run in duplicate and recovery rates of standard amount of epinephrine and norepinephrine are calculated for each analytical run. Recoveries up to at least 75% from biological materials have been reported.

2. ^{3}H-norepinephrine will be estimated by adding an aliquot of eluate (obtained after the alumina absorption of labelled amine as described above) in the counting solution (Instagel: Packard Instrument Co.) and the radioactivity will be determined in a Nuclear Chicago Scintillation counter.

3. ^{3}H-catechol deaminated metabolites will be assayed by the method of Kopin et al (J. Biol. Chem. 236: 2109, 1961).

4. ^{3}H-normetanephrine will be assayed by the method of Iversen et al (J.P.E.T., 150: 173, 1965).

5. ^{3}H-methylated deaminated metabolites will be estimated by the difference between the total radioactivity of the tissue extracts and the sum of other metabolites.

Enzyme Studies

Tissue will be removed, cleaned, weighed and homogenized in 2.0 ml of ice cold .25M sucrose. An aliquot (10 ul) of the homogenate will be

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used for assay of monoamine oxidase activity. The remaining homogenate will be centrifuged at 26000 g for 20 min. Aliquots of the clear supernatant fluid will be assayed for tyrosine hydroxylase, PNMT and COMT activities.

Monoamine oxidase activity will be assayed by measuring the conversion of ^{14}C-tryptamine to ^{14}C-indoleacetic acid as described by Wurtman and Axelrod (Biochem. Pharmacol. 12: 1439, 1964).

Catechol-o-methyl transferase (COMT) will be assayed by measuring the formation of ^{14}C-metanephrine on incubation with (-) epinephrine and ^{14}C-methyl-s-adenosylmethonine as described by Axelrod (in Method of Enzymology, Vol. 5, p. 748, 1959, New York Acad. Press).

Tyrosine hydroxylase activity will be assayed by the method of Levitt et al (J.P.E.T. 148: 1, 1965) with modifications described by Mueller et al (J.P.E.T. 101: 379, 1969).

Phenylethanol-N-methyl transferase activity will be assayed by the method of Axelrod (J. Biol. Chem. 237: 1657, 1962) using normetanephrine as the substrate and ^{14}C-S-adenosylmethionine will serve as a methyl donor.

Synthesis of norepinephrine in isolated tissues

The measurement of norepinephrine turn-over rate will be made by the amount of ^{3}H-norepinephrine formed from the ^{3}H-tyrosine according to the method of Weiner and Rabadjija (J. Pharmacol. Exp. Ther. 160: 61-71, 1968).

Many of these methods are already operative in our laboratory. The others will be set up for the purposes of this investigation.

Morphological Investigation

Complete autopsy will be performed in all experimental animals. In addition to a general survey of the histopathological changes of individual organs, and special attention will be paid in the study of the vascular changes in the heart, lung, kidneys, liver and endocrine glands.

Representative blocks of tissues from each organ will be placed into 10% neutral buffered formalin, Carnoy's fluid, and 100% ethanol respectively in order to carry out appropriate special stains in addition to a routine hematoxylin and eosin stains. The special stains utilized will include: Mallory's Azan stain, Periodic Acid-Schiff reaction, Verhoff Van Gieson stain and phosphotungstic acid hematoxylin stain. Appropriate blocks of tissues will also be frozen immediately to perform various enzyme stains.

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Electron Microscopy

Appropriate sections from heart, lung, liver, kidneys, endocrine glands will be fixed at specified times into 3% glutaraldehyde for one hour followed by Dalton's osmium fixative for one hour. The tissues will be embedded in Epon, sectioned and stained with uranyl acetate and lead citrate. Electronmicroscopy will be made with the use of RCA EMU 3 G Electronmicroscope III.

Analysis of Data

We have on the premises of the Department a D.E.C.-LINC computer for statistical analysis of the data. We will have a part-time computer programmer in the Department and if the need for special data analysis develops or if new programs are needed, he will be available to write and develop such programs. In this regard, it is anticipated he will be extremely helpful in developing programs to analyze the numerous samplings of the transmembrane potential before and after drug administration.

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SIGNIFICANCE OF RESEARCH

Cigarette smoking has been implicated by epidemiological studies as one of the major hazards to health in the United States. Not only has it been associated with respiratory diseases and disorders, but it is also implicated in the development of cardiovascular diseases, particularly in cases of hypertension and coronary diseases. So far, no causal agent has been found to explain these clearly established statistical relationships. It seems obvious that knowledge of causal factors or mechanisms of these diseases will be of great importance in the understanding of the disease process and in determining appropriate treatment.

Does smoking accelerate the development and intensity of hypertension and if so, is it a simple provoking mechanism superimposed on existing susceptibility? The intention of this investigation is to examine systematically the influence of smoking on the cardiovascular system in normotensive and hypertensive rats, and to correlate changes in catecholamine pattern with the onset and degree of initial and subsequent hypertension. In this way, we hope to provide 1) evidence of causal relationship between cigarette smoking and hypertension and 2) understanding of the mechanism involved in those pathological processes.

It is the ambitious long-term aim of this project to work toward the achievement of such a breakthrough, or at least to affect significant advances in the field of hypertensive therapy by the continued exploration of pathogenesis of experimental hypertension. We are more than hopeful that our efforts will aid in the elucidation of the role of smoking in the development of cardiovascular diseases, particularly hypertension. A successful approach in this area would make it possible to develop preventive measures and help place therapy on a more logical basis. This would in turn decrease the mortality rate. It is also hoped that our findings when added to an enormous pool of data being accumulated on a national and international scale may provide a key to the riddle of essential human hypertension.

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